

Brief Report

A Simple Method for Constructing E1- and E1/E4-Deleted Recombinant Adenoviral Vectors

HIROYUKI MIZUGUCHI¹ and MARK A. KAY²

ABSTRACT

We previously developed a two-plasmid *in vitro* ligation method that did not require a recombination step to produce new recombinant E1- or E1/E3-deleted adenoviral vectors. In this study, we have modified the system to improve the simplicity of vector construction and, in addition, to allow for production of an E1/E4-deleted vector.

INTRODUCTION

ONE OF THE PROBLEMS with the use of adenovirus (Ad) vectors has been the difficulty in the construction of new vectors. Several different protocols have been developed using various technologies to increase the ease of construction (Bett *et al.*, 1994; Ketner *et al.*, 1994; Chartier *et al.*, 1996; Crouzet *et al.*, 1997; He *et al.*, 1998). We described an *in vitro* ligation method (Mizuguchi and Kay, 1998) that used two plasmids for constructing first-generation Ad vectors. The vector plasmid has a complete Ad genome with a deletion of the E1 (and E3) region and three unique restriction sites, I-*CeuI*, *SwaI*, and PI-*SceI*, in the E1 deletion region. I-*CeuI* and PI-*SceI* are intron-encoded endonucleases (Marshall and Lemieux, 1991; Gimble and Thorner, 1992) that do not cut the Ad genome. The shuttle plasmid has a multicloning site between the I-*CeuI* and PI-*SceI* site. Both plasmids have a pUC-derived replication origin and ampicillin resistance gene. After the gene of interest is inserted into the multicloning site of the shuttle plasmid, the gene can be easily introduced into E1 deletion site of the vector plasmid at the I-*CeuI* and PI-*SceI* site. *SwaI* digestion of the ligation products prevents the production of a plasmid containing a parental Ad genome (null vector). Recombinant virus can be produced by the transfection of the *PacI*-digested, linearized plasmid into 293 cells.

In this study, we improve on the system by using a shuttle plasmid that has a kanamycin resistance gene instead of an

ampicillin resistance gene. Thus, no fragment isolation from a gel was required with the new shuttle plasmid. In addition, a series of shuttle plasmids with different promoters, and a series of vector plasmids for producing E1-, E1/E3-, and E1/E3/E4-deleted vectors, were constructed.

MATERIALS AND METHODS

Construction of recombinant plasmids

Vector plasmids pAdHM10 and pAdHM12 were constructed as follows. The *EcoRI/BglII* fragment of pEco-ITR0, which has the right end of the Ad type 5 genome (27331-right end) with a *BglII* site instead of a *BsgI* site, was ligated with the *EcoRI/MunI* fragment of pBHG11 (Microbix, Toronto, Ontario, Canada) after the *EcoRI/BglII* site of pEco-ITR0 and the *EcoRI/MunI* site of pBHG11 were changed into *NotI* sites by using a *NotI* linker, resulting in pEco- Δ E3,4. The *PacI* site in the E3 deletion region of pEco- Δ E3,4 was deleted (pEco- Δ E3,4P), and the *HpaI/SrfI* fragment of pEco- Δ E3,4P and pEco-ITR0 were ligated, resulting in pEco-ITR1. The *SrfI/ClaI* fragment of pEco-ITR1 was then inserted between the *SrfI* and *ClaI* sites of pAdHM1 (Mizuguchi and Kay, 1998), resulting in pAdHM9. pEco- Δ E3,4P was also cut with *SrfI* and *ClaI*, and ligated with *SrfI/ClaI*-digested pAdHM1, resulting in pAdHM11. The *ClaI* sites of pAdHM9 and pAdHM11 were

¹Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo 158-8501, Japan.

²Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA 94305.

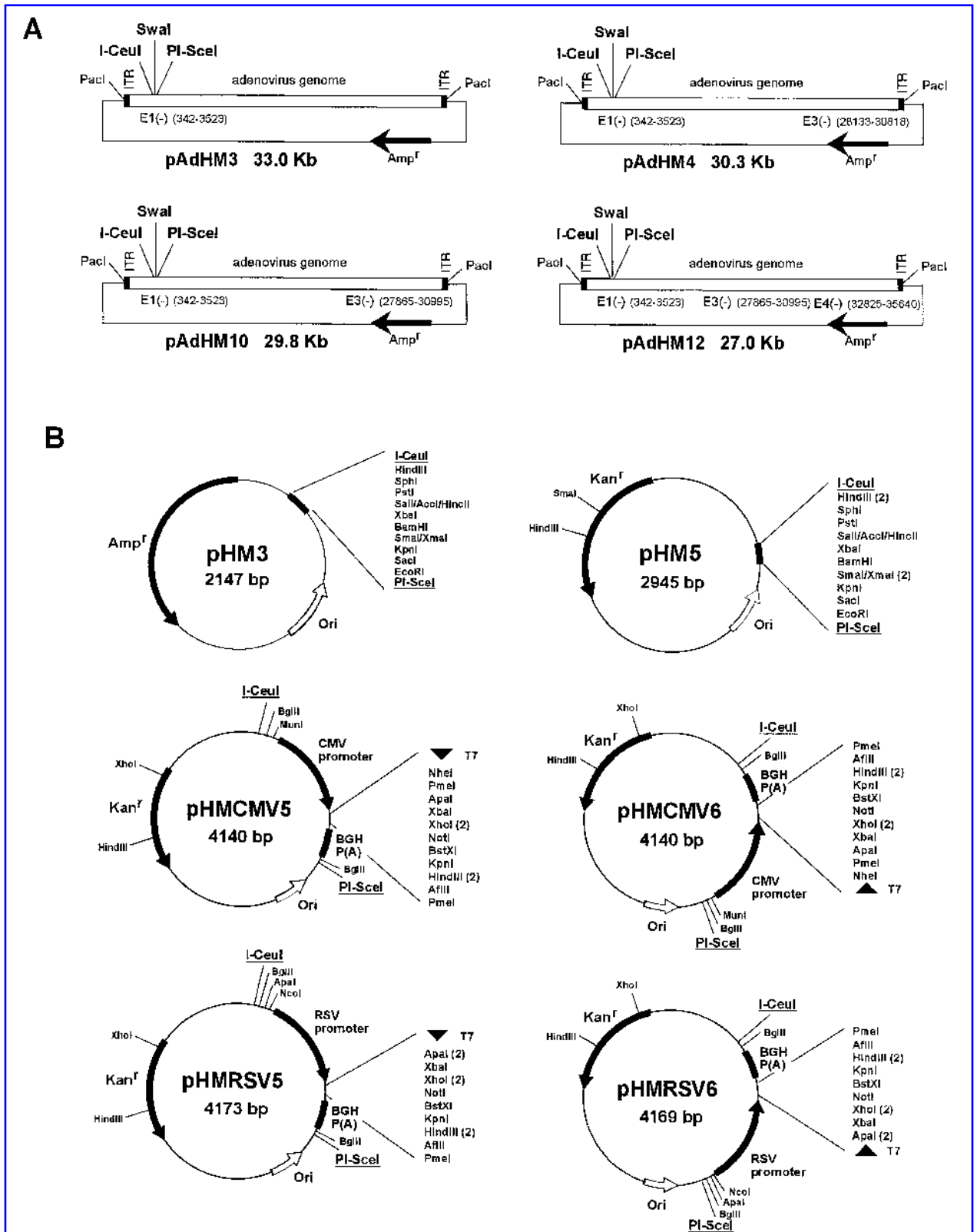


FIG. 1. The structure of the vector and shuttle plasmids. (A) Vector plasmids; (B) shuttle plasmids. Vector plasmids pAdHM3 and pAdHM4 and shuttle plasmid pHM3 were reported previously (Mizuguchi and Kay, 1998). The construction of the plasmids is described in Materials and Methods.

changed into *PacI* sites by using oligonucleotide 5' (5'-CGT-TAATTAA-3') (*PacI* recognition sequence is underlined). The resulting plasmids were named pAdHM10 and pAdHM12, respectively (Fig. 1A).

Shuttle plasmid pHM5 was constructed by ligation of the *DraI/SspI* fragment of pHM3 (Mizuguchi and Kay, 1998) with the *NheI/BamHI* fragment of pACYC177 (Nippon Gene, Toyama, Japan) after the *NheI/BamHI* sites were filled by the Klenow fragment of DNA polymerase (Fig. 1B). pHMCMV5,-6 and pHMRSV5,-6 were constructed by using pHM5 and the cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation signal from pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA) or the Rous sarcoma virus long terminal repeat (RSV LTR) enhancer/promoter from pREP10 (Invitrogen), respectively (Fig. 1B). Detailed information about the constructions is available from the authors on request.

Construction of E1/E3- or E1/E3/E4-deleted recombinant Ad vector DNA expressing β -galactosidase

Escherichia coli β -galactosidase (*lacZ*) gene derived from pCMV β (Clontech, Palo Alto, CA) was subcloned into the *XhoI/NotI* site of pcDNA3.1/Zeo, after the *SmaI* site of pCMV β was changed into a *XhoI* site by using a *XhoI* linker. The *lacZ* gene was then cloned by digestion with *NotI* and *XbaI* and inserted into the *NotI/XbaI* site of pHMCMV5, resulting in pHMCMVLacZ-1. pHMCMVLacZ-1, pAdHM10, and pAdHM12 were digested with *I-CeuI* and *PI-SceI*, and purified by phenol-chloroform extraction and ethanol precipitation. Digested pAdHM10 or pAdHM12 (0.1 μ g) was ligated with digested

pHMCMVLacZ-1 at 16°C for more than 2 hr. No agarose gel electrophoresis and fragment extraction were needed for both the vector and shuttle plasmid. To prevent the production of a plasmid containing a parental Ad genome, the ligation products were digested with *SwaI*, transformed with chemical-competent DH5 α (electrocompetent DH5 α can also be used), and grown in ampicillin, resulting in pAdHM10-LacZ and pAdHM12-LacZ.

Generation of recombinant virus

pAdHM10-LacZ and pAdHM12-LacZ DNAs were digested with *PacI* and purified by phenol-chloroform extraction and ethanol precipitation. To make an E1/E3- or E1/E3/E4-deleted Ad vector, linearized pAdHM10-LacZ or pAdHM12-LacZ was transfected into 293 cells or VK10-9 cells, which are 293 cells transformed with constructs containing the E4 transcription unit and pIX-coding sequences under the control of the inducible metallothionein promoter (Krougliak and Graham, 1995) (kindly provided by V. Krougliak, Mouni Sinai Medical Center, New York, NY), respectively. E1/E3-deleted virus was prepared as described previously (Mizuguchi and Kay, 1998). In the case of E1/E3/E4-deleted Ad vector, the cells were cultured with Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum and 10⁻⁶ M (final concentration) dexamethazone for 14 days. At this point, no cytopathic effect (CPE) was observed. The cell lysate prepared from the cells transfected by pAdHM12-LacZ was added to fresh VK10-9 cells. CPE was observed in about 7 days. Ad DNA was isolated from the cells with full cytopathic effect as described previously (Lieber *et al.*, 1996).

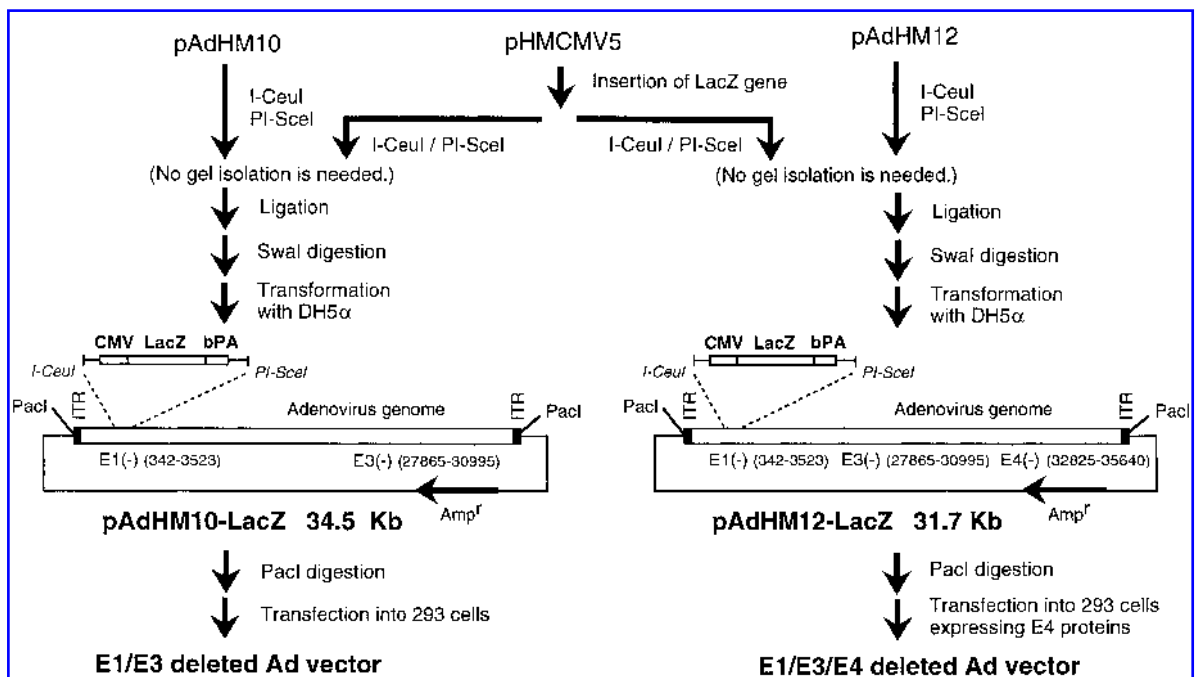


FIG. 2. Diagram of construction of E1/E3- or E1/E3/E4-deleted Ad vector expressing *lacZ*.

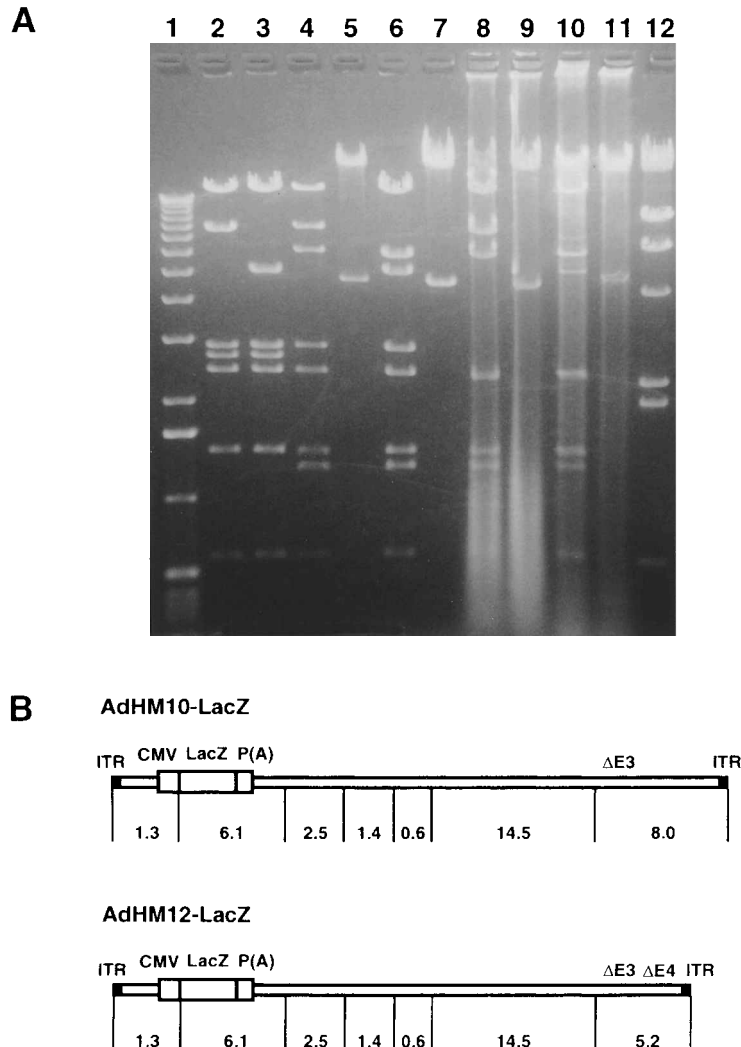


FIG. 3. Restriction endonuclease analysis. **(A)** Vector plasmid (pAdHM10, pAdHM12, pAdHM10-LacZ, pAdHM12-LacZ) or *lacZ*-expressing recombinant adenovirus DNA (AdHM10-LacZ, AdHM12-LacZ) was digested with *PacI/XhoI*, *XhoI*, or *I-CeuI/PI-SceI*, and run on a 0.8% agarose gel. Lane 1, 1 kb DNA ladder marker; lane 2, *PacI/XhoI*-digested pAdHM10; lane 3, *PacI/XhoI*-digested pAdHM12; lane 4, *PacI/XhoI*-digested pAdHM10-LacZ; lane 5, *I-CeuI/PI-SceI*-digested pAdHM10-LacZ; lane 6, *PacI/XhoI*-digested pAdHM12-LacZ; lane 7, *I-CeuI/PI-SceI*-digested pAdHM12-LacZ; lane 8, *XhoI*-digested AdHM10-LacZ viral DNA; lane 9, *I-CeuI/PI-SceI*-digested AdHM10-LacZ viral DNA; lane 10, *XhoI*-digested AdHM12-LacZ viral DNA; lane 11, *I-CeuI/PI-SceI*-digested AdHM12-LacZ viral DNA; lane 12, *HindIII*-digested λ DNA marker. A faint band at about 7 kb of *XhoI*-digested AdHM12-LacZ viral DNA (lane 10) is nonspecific, because *HindIII*-digested AdHM12-LacZ viral DNA contained only the expected fragments (not shown). **(B)** *XhoI* restriction map of the recombinant adenoviral DNAs. Fragment sizes (in kilobases) are shown below the genome. CMV, CMV immediate-early promoter/enhancer; P(A), bovine growth hormone polyadenylation signal.

RESULTS AND DISCUSSION

The ampicillin resistance gene of the shuttle plasmid pHM3 (Mizuguchi and Kay, 1998) was exchanged with a kanamycin resistance gene, resulting in pHM5 (Fig. 1B). This plasmid was used to derive shuttle plasmids that already have the CMV or RSV promoter and BGH polyadenylation signal in both orientations as shown in Fig. 1B. pAdHM10 and pAdHM12 were prepared for the construction of E1/E3- and E1/E3/E4-deleted vectors, respectively. pAdHM10 has a larger E3 deletion than

pAdHM4 (pAdHM10, 3.1 kb; pAdHM4, 2.6 kb). Up to 4.9, 7.6, 8.1, and 10.9 kb of exogenous DNA can be introduced into pAdHM3, -4, -10, and -12, respectively (Fig. 1A).

We constructed the CMV promoter-driven *lacZ*-expressing E1/E3- and E1/E3/E4-deleted Ad vectors as a representative example. The steps are diagrammed in Fig. 2. First, the *lacZ* gene was inserted into the multicloning site of pHMCMV5, resulting in pHMCMVLacZ-1. Second, pHMCMVLacZ-1 and vector plasmids (pAdHM10 and pAdHM12) were digested with *I-CeuI* and *PI-SceI*, and directly ligated without gel purification

of either the *lacZ* expression cassette or the vector viral sequence. Third, the ligation products were digested with *Swa*I, transformed with DH5 α , and selected with ampicillin. *Swa*I digestion cut parental vector plasmid, but not the expected recombinant plasmid. Only the ligated Ad plasmid DNAs with the *lacZ* expression cassette were selected. More than 75% of the transformants (seven of nine clones) had the correct insert as determined by restriction digestion. Fourth, recombinant plasmid pAdHM10-*LacZ* or pAdHM12-*LacZ* was cleaved with *Pac*I, and directly transfected into 293 cells or 293 cells expressing E4 gene product (VK10-9) (Krougliak and Graham, 1995), resulting in a homogeneous population of recombinant E1/E3- or E1/E3/E4-deleted vector expressing *lacZ* (AdHM10-*LacZ* or AdHM12-*LacZ*), respectively. DNA restriction analysis showed that vector plasmid with or without the *lacZ* expression cassette and viral DNA (AdHM10-*LacZ* or AdHM12-*LacZ*) contained the expected fragments (Fig. 3).

Finally, to confirm the functionality of the vector, HeLa cells were found to be equally transducible with either the AdHM10-*LacZ* vector or the AdHM12-*LacZ* vector as determined by X-Gal staining (data not shown).

Several systems for generating Ad vectors have been described; they include homologous recombination in mammalian cells (Bett *et al.*, 1994), yeast (Ketner *et al.*, 1994), or bacteria (Chartier *et al.*, 1996; Crouzet *et al.*, 1997; He *et al.*, 1998). The method we used to produce recombinant first-generation Ad vector requires molecular biology cloning techniques and no recombination steps in eukaryotic and/or prokaryotic cells. Here we modified this system by deleting the time-consuming process of gel purification of cloned DNA fragments, and constructed plasmids with various precloned cDNA-insertable expression cassettes. Moreover, the system described herein includes a plasmid to produce E1/E3/E4-deleted vectors. These types of modifications allow any laboratory to construct adenoviral vectors easily for gene transfer studies.

ACKNOWLEDGMENTS

We thank Dr. V. Krougliak for kindly providing VK10-9 cells. This work was supported by NIH-DK49022.

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Address reprint requests to:

Dr. Mark A. Kay
Department of Pediatrics and Genetics
Stanford University School of Medicine
300 Pasteur Drive
Room G305A
Stanford, CA 94305

E-mail: markay@stanford.edu

Received for publication March 20, 1998; accepted after revision May 20, 1999.

This article has been cited by:

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